Quantitative Gravimetric Fractionation of Brain Tissue

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With the procedure which is described, brain tissue is separated into six fractions, the sum of whose weights equals the dry weight of the tissue. Three of these primary fractions are further separated into subfractions. Recovery is quantitative at each step. The procedure, as applied to rat brain, gave consistent results; duplicate analyses of the two halves of brains, divided as equally as possible along the anteroposterior line, agreed closely, and the variation from rat to rat in the percentages of the various fractions in dry tissue was small, as illustrated by the following results: main lipid fraction, 40.65 ± 1.03 (S.D.) %; protein residue, 44.45 ± 0.87%; water-soluble fraction from Folch purification, 5.94 ± 0.23%; crude "strandin" fraction, 1.25 ± 0.05%. The average percentage of dry in fresh tissue was 24.87 ± 0.41. The method includes procedures for the separation of a fraction, obtained by treatment with acidified chloroform-methanol, into four well-defined fractions, of which one is characterized by extreme surface activity in aqueous solution. A small amount of lipid is so firmly bound in brain tissue as to resist extraction during exhaustive treatment with chloroform-methanol (2:1), acidified chloroform-methanol, and acetone. Significant radioactivity was present in most of the fractions from the brains of rats which had received a peritoneal injection of acetate-$C^{14}$ 5–15 months previously during the period of early development.

A remarkable metabolic stability of certain lipids in the adult brain has been reported by several investigators. Over 20 years ago, Waelsch et al. (1) found little or no labeling of the unsaponifiable lipids of the

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brain in adult rats whose body fluids had been enriched with deuterium, whereas under the same conditions there was marked labeling in very young rats (2, 3). These results indicated that the ability of the brain to synthesize cholesterol is lost in adult life. This interpretation was supported by the finding by several investigators of little or no labeling of cholesterol (1) in the brains of adult rats after feeding deuterium-labeled acetate (4) or after intraperitoneal injection of acetate-1-C\textsuperscript{14} (5–7); (2) in brain tissue from adult rats (8) or humans (9) after incubation in vitro with acetate-1, 2-C\textsuperscript{14}; or (3) in brains of adult cats after perfusion with octanoate-1-C\textsuperscript{14} (10). On the other hand, small but significant incorporation of C\textsuperscript{14} into cholesterol of adult rat brain occurred after injection of acetate-1-C\textsuperscript{14} into the cisterna magna by McMillan et al. (6), or into the cerebrum by Nicholas and Thomas (7). In unpublished experiments, Meltzer and I obtained similar results after injection of acetate-1-C\textsuperscript{14} into the subarachnoid space of adult rats by the technic of Lindberg and Ernster (11). The apparent discrepancy between these and the earlier results has not been explained, but the finding by McMillan et al. (6) of no decrease in the specific activity of brain cholesterol thus labeled up to 72 days after the injection suggests that the labeling resulted from synthesis of cholesterol in the process of growth, which continues throughout life in the rat.

Further important evidence for the metabolic stability of cholesterol and of other lipids in the adult brain has been supplied by Davison and his colleagues in a series of studies in which labeling was induced in the brain by administration of cholesterol-4-C\textsuperscript{14} (12–14), DL-serine-3-C\textsuperscript{14} (15), P\textsubscript{i}\textsuperscript{32} (16–18), or glycerol-1-C\textsuperscript{14} (18) during the period of early development when growth of the brain and myelination are proceeding rapidly in chicks, rats, and rabbits. In all of these experiments the labels persisted in the brain lipids at relatively high levels well into adult life. Since the lipids thus shown to be metabolically stable, particularly cholesterol, cerebrosides, and sphingomyelin, are in all probability major components of myelin, Davison et al. (13) concluded: "The uniform persistence of these three myelin lipids over this long period is consistent with the belief that when once deposited in the myelin sheath at the time of its formation, they undergo little subsequent 'turnover'; the myelin sheath must, therefore, be regarded as one of the more permanent tissue elements."

This concept is of such manifest importance that it is worthy of further study, and more than 2 years ago I undertook an investigation similar to those of Davison and his colleagues but with a somewhat
different approach. Instead of aiming at the labeling of a particular lipid, the plan was to induce labeling of as many components of the brain as possible by administering acetate-C¹⁴ to rats during early development and to determine the distribution of the isotope in the brain at various intervals thereafter. For this purpose, a procedure for quantitative, gravimetric fractionation of brain tissue was required. The description of such a procedure is the primary purpose of this communication.

Method and Materials

Special Apparatus

*Tissue Homogenizer* The apparatus* (Fig. 1 and 2) is similar to the tissue disintegrator which was previously described (19, 20), but it is made of Teflon except for the piston assembly, which is of stainless steel.

*Funnels with Fritted Discs Pyrex No. 37730F* Equipped with glass stoppers as shown in Fig. 3 and 4, these are calibrated as follows. The funnel is inverted and the space under (now above) the filter and the stem are filled with water. The stopper is inserted in the stem and the funnel is clamped in an upright position. Water (4 ml.) is added, and the height above the lower edge of the fritted disc is measured with a vernier caliper. Readings are taken at 1-ml. intervals to 8 ml. and plotted on coordinate paper.

*Centrifuge tubes, 40 ml., graduated, Pyrex No. 8144* Stoppers are ground as previously described (19) and attached with fine nichrome wire.

*Flasks, 50 ml., round-bottom, with standard taper 24/40 joints* These are equipped with nooses made of fine nichrome wire to permit hanging in a balance.

Solvents and Solutions

All solvents, except chloroform, are distilled through a 40-plate Oldershaw column and tested from time to time for absence of residue. Chloroform is distilled through a short column from K₂CO₃ into sufficient ethanol to make the final concentration 1%.

*Chloroform-Methanol (2:1, v/v)* [later described as C-M]

*Chloroform-Methanol (1:2, v/v)*

*Ethanol, 50%*  

*Buchler Instruments, Inc., Fort Lee, N. J.*
Fig. 1. Tissue homogenizer, disassembled. Dimensions of Teflon cups are: inside diameter, 2 cm.; inside height, 2.85 cm.; thickness of wall, 4 mm. Clearance between stainless steel piston and cylinder is 0.007 in. Height of piston is 9 mm. Bottom face of cylinder, upper face of cup wall, inner faces of head and caps, and top and bottom faces of piston are machined to smooth, plane surfaces which are perpendicular to vertical axis. Fig. 2. Tissue homogenizer, assembled. Fig. 3. Insertion of stopper in stem of funnel at Step A. Manner of holding the funnel and spatula during washing of space under filter is also shown. Fig. 4. Assembly of apparatus during incubation at Step B.

Buffered 0.5 M NaCl Solution in Upper Phase (22) NaCl (6.084 gm.) and 0.583 gm. of Tris are dissolved in about 70 ml. of water and titrated with N HCl to pH 8.8. The solution is transferred quantitatively to a 100-ml. volumetric flask and made to volume; 94 ml. are mixed with 96 ml. of methanol and 6 ml. of chloroform.

Acidified Solvent (23) Chloroform-methanol concentrated HCl (20:10:0.1, v/v/v).

General

Wherever applicable, previously described technics (19, 20) are used. Transfer apparatus (Fig. 2C in Methods of Biochemical Analysis (19)) is made with corks sprayed with Teflon aerosol (Fluoro-Glide)* instead of rubber stoppers.

Protection Against Oxidative Degradation

Removal of solvents is carried out either by lyophilization, or in a rotary evaporator, or in a stream of pure nitrogen at about 45° (19). Without delay, dried samples are placed over Drierite† and paraffin in a vacuum desiccator which is evacuated with an efficient oil pump. Pure nitrogen is admitted and the desiccator is evacuated; this process is repeated once more. The extreme precautions described by Rouser et al. (24) (use of oxygen-free solvents, etc.) were not considered to be essential to the present study and were not applied, but they could be if desired. Rouser et al. (25) obtained essentially the same results whether or not extraction of lipids from beef brain was carried out in the absence of oxygen.

Weighing

Since the success of this procedure depends on the precision of weighing, the technic used is described in detail. Semimicro, Sartorius Selecta balances are used for all weighings. The Teflon cups and caps (Fig. 1) are washed carefully with water and methanol and thoroughly dried before weighing. Glass apparatus to be weighed is carefully cleaned, usually with sulfuric acid-chromate or by ultrasonic

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*Scientific Glass Apparatus Co., Bloomfield, N. J.
†Fisher Scientific Co., New York, N. Y.
treatment. It is filled with tap water and emptied at least 3 times and rinsed with distilled water 3 times. After drying, the apparatus is rinsed 2 or 3 times with small amounts of C-M and clamped in an inverted position until dry. The outer walls of flasks and centrifuge tubes are rinsed with distilled water and wiped carefully with a clean, lint-free towel. Funnels are wiped first with a moist towel and then with a dry towel. After wiping, apparatus is handled only with an hemostat or tongs; it is kept in vacuo over Drierite and paraffin overnight. Small apparatus (5-ml flasks and filter funnels) is equilibrated in Dri-Jar desiccators (26) for about 0.5 hr, before weighing. Large apparatus is transferred to the balance from the vacuum desiccator. The same timing and sequence are used for the tare and gross weighings. A stopwatch is started when the balance is released, and the weight is read at a time determined by the characteristics of the balance (30-35 sec. with the balances used in this work). The zero point is read after each weighing at the same time interval, and the weight is corrected by one-half of any deviation. With every one to four pieces of apparatus, one of the same type and size is weighed as a control, and the net weights are corrected for any change in this weight. With small apparatus such as 5-ml flasks this correction is small, usually <0.05 mg., rarely >0.1 mg., but with large apparatus such as 50-ml flasks it may be quite large, 0.5 mg. or, rarely, even more. In some instances this correction has been larger than the net weight. The inclusion of control weights is essential to the achievement of the precision shown under Results and Discussion. It is also essential with large apparatus for the following reason: In weighing small apparatus, the balance comes to rest in about 0.5 min. and does not change for several minutes, but with large apparatus, after apparent rest is reached at 30-35 sec., a slow drift in the direction of a smaller weight begins and continues for 4-6 min. It has been impossible to relate this phenomenon to the presence of moisture or to electric charge. To wait 4-6 min. for the balance to come to rest would require an inordinate amount of time in weighing, and would increase the risk of oxidation through exposure of samples to air. In a large series of weighings it was noted that the amount of drift was nearly the same for all of the members of a set, including the control. This makes it possible to record the weights at 30-35 sec. and to apply the control correction. Weights thus obtained agreed very closely with those obtained in the same flasks by waiting for the balance to come to rest.
Procedure (Fig. 5)

Step A

Funnels and centrifuge tubes are tared and assembled as shown in Fig. 6. The tissue to be analyzed (one-half of a rat brain in the present work) is placed as rapidly as possible after killing the animal in a tared Teflon cup, the cover is screwed on tightly, and the cup is weighed. Methanol (4 ml.) is added, and the homogenizer head (Fig. 1) with the piston at its uppermost position is screwed on and tightened with two large pliers. The piston is forced as far down as it will go, twisted slightly, and pulled to the top, again with a twist to force tissue from between the surfaces of the piston and the Teflon head. This process is repeated briskly for about 35 complete strokes, an arbitrary number found to give good homogenization. At the last stroke, the piston is pulled tightly against the head, and the apparatus is hung by the handle in a spring clamp. The cup is unscrewed with care to keep it in a
vertical position, and the homogenate is transferred to the funnel with gentle suction, as shown in Fig. 6. Immediately after the transfer is finished, the suction is relieved by opening a spring clamp on a large, rubber, side tube. If there is delay at this point, tissue will dry on the inside of the transfer tube; such dried tissue cannot be removed. A 1-ml. beaker is held under Tube S (Fig. 2C (19)) and the outside is rinsed with two or three drops of methanol. This rinse is sucked over by closing the rubber side tube momentarily with the fingers. The piston assembly is removed from the clamp, and the piston is pushed down about ¾ in. and held at an angle over the cup. With a transfer pipet (Fig. 2A (19)) about 0.5 ml. of methanol is run over the piston to wash some, but not necessarily all, of the tissue which usually adheres to it into the cup. The head is screwed on as before and the piston is operated for five strokes. The homogenate is transferred as before. This washing procedure is repeated 3 more times. After the last wash the inner end of Tube S is rinsed with two or three drops of methanol, and a little methanol is sucked over from the 1-ml. beaker. Usually, a few

Fig. 6. Apparatus assembled for Step A. Procedure for transfer of homogenate from Teflon cup to funnel is shown. In this and Fig. 3, 4, and 7, apparatus was not photographed in actual use because of technical difficulties.
particles of tissue remain on the Teflon head and piston, and occasionally in the cup, and there is a little dried tissue on the outside of the transfer tube. After the chloroform has been added (see below), this tissue is transferred to the funnel with the aid of a small metal spatula. Without delay, the height of liquid in the funnel above the lower edge of the fritted disc is measured with a vernier caliper, and the volume is determined from the calibration chart. After subtraction of the calculated volume of water from the tissue (wt. in grams \( \times 0.75 \)), twice the volume of chloroform is added. Stirring is not necessary. The funnel is loosely stoppered, and filtration is allowed to proceed by gravity for about 3 hr., after which time the major part has usually filtered.

Filtration of the remaining suspension is accelerated by the application of gentle suction, which is continued until most of the solution has filtered; the suction is relieved before the cake of tissue has been sucked dry. The wall of the funnel is washed with about 0.8 ml. of C-M, added with a transfer pipet. The tissue is stirred into uniform suspension with a small porcelain spatula. Suction is applied until most of the solution has filtered and the cake appears to be moist. This washing procedure is applied 9 more times. If necessary, at about every third wash, the solvent is ejected onto the spatula to free it from large masses of tissue suspension. After the tissue has been stirred into suspension at the tenth wash, the spatula is freed of adhering tissue by forceful washing with a little more C-M.

The filter assembly is removed from the centrifuge tube, the spatula is raised a little, it and the funnel are grasped with one hand, and the assembly is tipped back far enough (Fig. 3) to permit a small amount of C-M to be injected into the stem with a fine-tipped pipet. The space under (now above) the filter is rinsed by rotating the assembly. The filter is tipped up so the C-M will run into the centrifuge tube. The outside of the stem is rinsed with a little C-M. This washing procedure is repeated twice more. After the last wash, the space above the filter is filled with C-M, the stopper is tightly inserted in the stem (Fig. 3), the outside of the stem and stopper are rinsed with C-M, and the assembly is transferred to another centrifuge tube.

Step B

About 4 ml. of acidified solvent are added to the funnel and the tissue is stirred into a uniform suspension with care to break up all lumps. Pigment is extracted from the tissue, which becomes almost pure white. The spatula is lifted to the mouth of the funnel, and any tissue
adhering to it is scraped loose with a small metal spatula. Both spatulas are rinsed with acidified solvent. This process is repeated until no white residue is left on either spatula. About 2 ml. of acidified solvent, applied in 4 or 5 portions with a small transfer pipet, are used in washing. The assembly (Fig. 4) is placed in an incubator at about 38°, and, after equilibration for 0.5 hr., the funnel is stoppered and left over night.

The assembly is removed from the incubator, the upper stopper is removed, and the funnel is raised in the clamp so the lower stopper is just above the mouth of the centrifuge tube. The stopper is grasped with a small hemostat which has been rinsed with C-M. The funnel is rotated gently in the clamp until the stopper is loosened. While still over the centrifuge tube, it is rinsed with a little C-M. The funnel is lowered, the cork is tightened, and gentle suction is applied. Filtration and washing are carried out as in Step A except that only six washes with C-M are applied. The space under the fritted disc is rinsed as in Step A.

**Step C**

The funnel is transferred to a tared 25-ml. suction flask (Fig. 7), about 3 ml. of acetone are added, and the tissue is stirred into suspen-
sion. The solvent is filtered, and the tissue is washed 5 times with 2-ml. portions of acetone, the tissue being stirred into suspension with the suction off at each wash. Before the last wash is filtered, the spatula is freed of adhering tissue as described in Step A.

The acetone is evaporated from the filtrate with a stream of nitrogen (19), and the residue is weighed (Fraction 1). Acetone is evaporated from the residue in the funnel with a gentle stream of nitrogen, and the residue is weighed (Fraction 2).

Step D

C-M is added to the filtrate from Step A to the 30-ml. mark. Water (7.5 ml. minus the weight of tissue, in grams) is added, the stopper is moistened with water and inserted tightly, and the contents are equilibrated by slow inversion and reversion 30 times and allowed to stand over night. The tube is centrifuged 5 min. at <1500 rpm. As much as possible of the upper phase is sucked with a Van Slyke-Rieben (26) pipet into a tared 50-ml. flask. This is best done by clamping the tube on the rod of a Lab-jack and raising it until the horizontal tip of the pipet is about 2 mm. above the interface. The suction should be just sufficient to start siphoning. Good illumination should be used and care should be taken to avoid any transfer of lower phase. Five milliliters of PSUP are added slowly down the wall of the tube which is rotated so the entire wall is washed. There should be no mixing with the lower phase. The upper phase is stirred gently with a small metal spatula to mix the residual upper phase with the PSUP. The spatula is rinsed with 2-3 drops of PSUP, and the upper phase is sucked off as before. This washing procedure is repeated 4 more times.

The upper phase is concentrated with a stream of nitrogen to about 10 ml., lyophilized, and weighed (Fraction 3).

Step E

Twenty milliliters of NaCl solution in buffered PSUP are added to the lower phase from Step D, the phases are equilibrated, the tube is allowed to stand over night and centrifuged, the upper phase is removed, and six washes with PSUP are applied, all as described in Step D. The upper phase is discarded.

Step F

Precipitate in the form of a rubbery "skin" should appear at the interface in Step E. With a fine wire this is teased and pulled up onto the wall. The clear solution is sucked into a tared 50-ml. flask. As
surface of the liquid is lowered, any precipitate which may have re-
mained at the interface adheres to the wall. The tube is washed 5 times
with small portions of C-M (19). The precipitate is not dislodged by
the solvent which runs down the wall during washing. The centrifuge
tube is dried and weighed (Fraction 4).

The lower phase is evaporated at <45° with a stream of nitrogen
(19). When the volume reaches 2 or 3 ml., evaporation becomes very
slow. A film of lipid forms over liquid which is largely aqueous. At
this point about 5 ml. of acetone are added and the flask is swirled to
form a milky emulsion. Evaporation is continued until most of the
acetone is gone. This process is repeated with smaller amounts of
acetone until the residue appears to be dry. Small amounts of acetone
are added to the dry-appearing residue and evaporated twice more.
The residue is weighed (Fraction 5).

Step 6

Immediately after weighing Fraction 5, about 1 ml. of C-M is added,
and the flask is stoppered. A precipitate, presumably of proteolipid
protein (PLP), remains undissolved. The suspension is transferred
with a small transfer pipet (Fig. 2A (19)) drawn to a fine tip to a tared
filter funnel (Pyrex No. 36290M), which is arranged to feed into a
tared 5-ml. volumetric flask by means of a 2-holed stopper and a short
piece of rubber tubing. The other hole of the stopper carries a glass
tube through which suction may be applied. The 50-ml. flask is washed
5 times with small portions of C-M. No attempt is made to transfer all
of the precipitate to the funnel. The bulb is removed from the transfer
pipet, which is rinsed 3 times with small amounts of C-M. After the
transfer is complete, suction just sufficient to induce slow filtration is
applied. There is danger that the filter may clog if the suction is too
great. If this should happen, the sample may be saved by scraping the
precipitate from the filter with a small metal spatula. The funnel is
washed 5 times with small portions of C-M.

The funnel and the 50-ml. flask are dried and weighed (Fractions 6
and 7, respectively). The solution in the 5-ml. flask is taken to dryness
with a stream of nitrogen and with the aid of additions of acetone as
dryness is approached. After weighing, Fraction 8, which is the main
lipid fraction, should dissolve completely in C-M.

Step H

Fraction 3, the residue from the upper phase from Step D, is dis-
solved in a minimal amount of water and transferred with a small
transfer pipet and five washes to a dialysis sack prepared as follows. A length of wet 15/16 in. (flat width) Visking tubing is passed onto a piece of 15-mm. glass tubing, about 5 cm. long, constricted at the lower end to about 10 mm., flared at the upper end, and equipped with a long piece of fine nichrome wire. The Visking tube is secured to the glass tube with a rubber band and securely tied with a double knot to form a sack which extends 4-5 cm. below the glass support. The excess tubing is cut off at the knot. The sack is filled with water, suspended by means of the nichrome wire in water in a large flask, and boiled for several hours. Just before use, the sack is hung by means of the glass tube in a spring clamp and washed several times by filling with water and sucking the water out. The outside is also copiously washed. In transferring the sample, care is taken to pass the tip of the pipet well into the sack before the solution is expelled. The bulb is removed, and the pipet is rinsed with water.

About 280 ml. of water are placed in a thoroughly cleaned 600-ml. Erlenmeyer flask. The sack is lowered into the flask until the surface of the solution in it is at a level with the surface of the water and secured in that position by means of the nichrome wire and a cork protected with aluminum foil. Dialysis is allowed to proceed at 4° for 2 or 3 days.

The sack is suspended in a spring clamp, and the solution is transferred by suction to a tared 50-ml. flask. Washing is carried out with a polyethylene wash bottle whose flexible nozzle is inserted through the glass support tube and turned so the entire inner surface of the sack is rinsed with a small amount of water which is sucked into the flask. This is repeated several times. The solution is lyophilized and the residue is weighed (Fraction 9).

The dialysate is transferred to a 1-L., round-bottomed flask which has been thoroughly washed after standing at least 24 hr. full of H₂SO₄-chromate. The solution is concentrated with a rotary evaporator to about 1 ml. and transferred by suction to a tared 5-ml. volumetric flask. Washing is carried out as previously described (19) with 50% ethanol. At the first wash, the flask is turned so the solvent (about 1 ml.) wets the entire surface. If the flask has been well cleaned, this is easily accomplished, and the following four washes run smoothly down the wall of the flask in a continuous film as the flask is rotated. With care, the total volume can be kept within 4 ml. Occasionally, some concentration of the solution in the 5-ml. flask may be necessary before the
washing is completed. The solvent is removed with a stream of nitrogen and the residue is weighed (Fraction 10).

Step I

The filtrate from Step B is transferred to a tared 50-ml. flask (19), concentrated with nitrogen at <40° to about 6 ml. (the original volume of acidified solvent), and lyophilized as follows. After being attached to the apparatus, the flask is partly immersed in a Dry Ice-ethanol bath in a beaker which is supported on a Lab-jack. After 5–10 min., the pump is started, and the bath is lowered when the pressure has been reduced to 2 mm. or less. The residue is weighed (Fraction 11).

Step J

Immediately after weighing, 2 ml. of C-M (1:2) are added to Fraction 11, a stopper is inserted, and the flask is allowed to stand for at least 2 hr. The suspension is transferred with the technic described in Step G to a tared 12-ml. centrifuge tube and centrifuged 5 min. at 1500 rpm. The clear, colored, supernatant solution is transferred with the pipet used in the previous transfer to a tared 5-ml. volumetric flask. A small amount of C-M (1:2) is added, the precipitate is stirred into suspension, and the centrifuging and transfer are repeated. This washing procedure is repeated 4 more times. The solution is concentrated in the flask with nitrogen to make room, if necessary. Drying is completed with nitrogen (19), and the residue is weighed (Fraction 12). The flask and centrifuge tube are dried and weighed to give Fractions 13 and 14, respectively.

Step K

Chloroform (1-2 ml.) is added to Fraction 12 and the flask is stoppered and allowed to stand 1-2 hr. The clear, highly colored solution is transferred with 5 washes with chloroform to a tared 5-ml. flask with the technic described at Step G. The solvent is removed with nitrogen and the residue is weighed (Fraction 15). The chloroform-insoluble residue is dried and weighed (Fraction 16).

Step L

Water (about 1 ml.) is added to Fraction 13 (in the 50-ml. flask) and the flask is stoppered and placed in the refrigerator until Step K has been completed. The water, which usually remains clear, is transferred with 5 small washes to the centrifuge tube. (All transfers in this Step are carried out with the technic described at Step G.) Water (about 1 ml.) is added to Fraction 16, most of which dissolves. This solution (or
suspension) is also transferred with 5 washes to the centrifuge tube. The contents of the centrifuge tube are stirred with a small metal spatula to suspend Fraction 14. The tube is centrifuged and the clear supernatant solution is transferred to a tared 50-ml flask. A small amount of water is added to the centrifuge tube, the residue is stirred into suspension, the tube is centrifuged, and the supernatant solution is transferred to the 50-ml flask. This washing procedure is repeated 4 times more. The solution in the 50-ml flask is lyophilized and the residue is weighed (Fraction 17). The residues in the 5-ml flask, 50-ml flask, and centrifuge tube are dried and weighed to give Fractions 18, 19, and 20 respectively.

Step M

The water-soluble Fraction 17, after weighing, is dialyzed, and the nondialyzable fraction and dialysate are treated as described at Step H and weighed (Fractions 21 and 22, respectively).

Notes on Procedure

Step A

The few particles of tissue which usually resist homogenization and transfer appear to be connective tissue. The amount appears to be very small, and its loss would have little effect on the results except possibly on dry weight.

During the time required for the transfer of the homogenate and washes, no filtration has been observed. Liquid does penetrate the fritted disc but the amount is judged to be too small seriously to affect the measurement of volume. That the desired ratio of chloroform to methanol (2:1) is achieved is shown at Step D. The volume of lower phase has always been between 21 and 22 ml. (in most experiments, between 21.5 and 21.9 ml.), as expected (21).

The suspension is allowed to filter by gravity for about 3 hr. for two reasons: (1) If suction is applied at once, there is some danger that the filter will be clogged; (2) it is possible, but not proved, that extraction of lipids is improved by the longer contact between tissue and solvent.

The exhaustive washing procedure was adopted on the basis of an extensive series of experiments in which the amounts of material removed by various sizes and numbers of washes were measured. Even after 10 washes, as described, very small amounts of weighable material are extracted by further washing.

The washing of the space under the filter is applied to guard against loss due to channeling during filtration. The moist tissue adheres
quite firmly to the filter and there is little danger of loss if the funnel is not tipped back too far.

**Step B**

The amount of tissue adhering to the spatula is minimized by the rinse at the tenth wash in Step A.

Leakage into the centrifuge tube during incubation has not exceeded 0.5 ml.

**Step C**

The treatment with acetone is carried out primarily to facilitate drying of the protein residue. Surprisingly, after the exhaustive treatment with solvents in Steps A and B, acetone always extracts weighable material, but the amount is small and it has not been further investigated.

**Step D**

The amount of water to be added is calculated according to Folch *et al.* (21).

Excessive agitation during equilibration may result in emulsions which are difficult to break.

The centrifuge should be at ambient temperature; if it has been heated by previous use the phases may become cloudy on standing at room temperature. There is usually a thin film of material (emulsion or fluff †) at the interface after centrifuging. The volume of the lower phase should be 21–22 ml.

**Step E**

There should be no change in the volume of the lower phase during the treatment with the NaCl solution.

In two experiments, the upper phase and washes were taken to dryness with a rotary evaporator, and the residue was dissolved in a small amount of water and dialyzed. No weighable residue (after correction for the blank) remained in the dialysis sack. This result shows that there is no loss of nondialyzable material from the tissue, and the upper phase from the NaCl treatment is, therefore, discarded. It may be assumed that all dialyzable material was removed at Step D.

**Step F**

In some experiments, sufficient methanol (about 3 ml.) was added to cause the small amount of residual upper phase to blend with the lower phase. This probably is not necessary since a two-phase system forms as the volume is decreased.
Attempts were made to evaporate the lower phase with a rotary evaporator, with the Rotary Evapo-Mix,* or by lyophilization. All failed because of excessive foaming or explosive boiling as dryness was approached.

The treatment with acetone is applied primarily to facilitate drying, but it may increase the release of PLP (see below).

**Step G**

There is a large, fortuitous variation in the relative amounts of precipitate which go into suspension (Fraction 6) and adhere to the flask wall (Fraction 17).

The transfer, filtration, and washing can easily be accomplished with a total volume of 3–4 ml. of C-M with the technic previously described by the author (19).

It is difficult to remove C-M completely from concentrated solutions of lipids. (Fraction 8 from one-half a rat brain contains about 100 mg.) The addition of acetone facilitates drying of such solutions.

**Step H**

During the development of the procedure, a large number of blank dialyses was carried out. Despite all attempts to insure cleanliness, including excessive soaking and washing of the dialysis sacks, small but definite residues were always found, the amount in the small volume of water in the sack being almost as large as that in the large volume of “dialyzate.” Boiling the sacks considerably reduced the blank, but weighable residues were still present. For the greatest precision, especially when the amounts of material being dialyzed are small, it is desirable, therefore, that blank dialyses be carried out and that corrections for the residues found be applied.

Fraction 9, largely strandin, should appear as a white, fluffy residue, a small weight of which occupies a large volume.

**Step I**

In all of the experiments so far carried out, the filtrate from Step B was transferred to a 25-ml. volumetric flask, and, after dilution to volume and mixing, 5 ml. were pipetted for analyses. The pipet was rinsed into the centrifuge tube and the remaining 4/5 of the solution were transferred to a tared 50-ml. flask as described. The use of a centrifuge tube to receive the filtrate was continued from earlier experiments, where a Folch purification, similar to the procedure described in Step

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D, was applied. With the procedure as described, it might be better to filter directly into a 50-ml. tared flask.

In some experiments, C-M (1:2) was added to the dry residue, and the lyophilization was repeated to insure removal of HCl. No effect on the results was observed, and this procedure is probably unnecessary.

Step J

With the technic described, the solvent leaches material, including most, or all, of the reddish pigment, from a gelatinous residue which adheres to the wall of the flask. Little of the insoluble material is transferred to the centrifuge tube. In some experiments, the residue was scraped into suspension with a bent metal spatula so a large part of the insoluble material was transferred. No effect on the results was observed.

Step K

No precipitate has gone into suspension in chloroform under the described conditions in any of the experiments which have been carried out. The clear, highly colored solution is easily separated from the insoluble material.

Step L

The purpose at this step is to combine in one fraction all the water-soluble material extracted by the acidified solvent. Most of it is in Fraction 16, and it is not certain that any remains in Fractions 13 and 14; but to be sure, treatment with water is carried out as described.

Step M

Fraction 17 is exceedingly surface active, and care must be taken to avoid excessive foaming in the transfer to the dialysis sacks. Fraction 21, during lyophilization, should appear much as strandin does, i.e., as a fluffy material which occupies a large volume in relation to its weight. It is highly aquaphilic and surface active.

Results and Discussion

In each of the 36 experiments which have been carried out in the development of the procedure, a rat brain was divided along the antero-posterior mid-line into two parts, as nearly equal as possible, and these were analyzed side by side with two objectives: (1) When the same technic was applied to both, the results gave a measure of the re-
prodicibility of the procedure. This is illustrated by some of the data from a typical experiment (Table 1). (2) The effects of changes in technic, applied to one of the half brains, were tested. This is illustrated by the study of procedures for isolation of PLP (see below).

**Dry Weight**

In evaluating the procedure, particular attention was paid to the percentage of dry weight in fresh tissue since it is calculated from the sum of several weights (primary fractions) and would be expected, therefore, to reflect errors and inaccuracies. The results, as typified in Table 1, were satisfactory, especially so, in view of the expectation that there would be some differences between the results obtained on the two halves of a brain due to failures to divide the brain into exactly equal parts. An appreciable difference in the proportions of white and grey matter would have a considerable effect on the percentage of dry weight. In seven experiments carried out with the procedure essentially as described above, except for the omission of Steps E and F in the analysis of one half-brain (H-2) (see below for explanation), the average dry weight percentages for H-1 and H-2 were 24.86 and 24.88, respectively. The maximal difference between the two values was 1.03%, and the average difference was 0.34%.

**Table 1. Primary* Fractions from a Rat Brain (Experiment 35)**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Weights mg.</th>
<th>In fresh tissue</th>
<th>In dry tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-11</td>
<td>H-21</td>
<td>H-1</td>
</tr>
<tr>
<td>1 Acetone extract</td>
<td>0.32</td>
<td>0.27</td>
<td>0.03</td>
</tr>
<tr>
<td>2 Protein residue</td>
<td>109.93</td>
<td>104.86</td>
<td>11.13</td>
</tr>
<tr>
<td>3 Water-soluble</td>
<td>14.25</td>
<td>13.45</td>
<td>1.44</td>
</tr>
<tr>
<td>4 PLP*</td>
<td>2.12</td>
<td>---</td>
<td>0.21</td>
</tr>
<tr>
<td>5 Main lipid + PLP</td>
<td>106.76</td>
<td>104.50</td>
<td>10.81</td>
</tr>
<tr>
<td>6 Total dry weight††</td>
<td>250.41</td>
<td>240.82</td>
<td>25.36</td>
</tr>
</tbody>
</table>

*The fractions whose sum gives the dry weight are designated as primary.
†Numbered arbitrarily in the order in which they are described in the text.
‡Halves of the brain.
§Weights of fresh tissue were: H-1, 987.36 mg.; H-2, 945.08 mg.
∥Upper phase from Folch purification.
*Released by 0.5M NaCl at pH 8.8.
**Steps E and F not applied in H-2.
††Extracted by 0.033N HCl in C-M.
††Sum of preceding fractions.
Table 2. Percentages of Protein Residue (Fraction 2) in Wet and Dry Tissue

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Percentage in wet tissue</th>
<th>Percentage in dry tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-1</td>
<td>H-2</td>
</tr>
<tr>
<td>29</td>
<td>11.09</td>
<td>11.54</td>
</tr>
<tr>
<td>30</td>
<td>10.97</td>
<td>11.03</td>
</tr>
<tr>
<td>31</td>
<td>10.87</td>
<td>10.87</td>
</tr>
<tr>
<td>32</td>
<td>10.92</td>
<td>10.92</td>
</tr>
<tr>
<td>33</td>
<td>11.13</td>
<td>11.09</td>
</tr>
<tr>
<td>35</td>
<td>11.13</td>
<td>11.10</td>
</tr>
<tr>
<td>36</td>
<td>11.22</td>
<td>10.88</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>11.05</td>
<td>11.06</td>
</tr>
</tbody>
</table>

*Experiment 34 was lost in attempts to evaporate at Steps E and G in a Rotary Evapo-mix.

Table 3. Percentages of Main Lipid Fraction (8) in Wet and Dry Tissue

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Percentage in wet tissue</th>
<th>Percentage in dry tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-1</td>
<td>H-2</td>
</tr>
<tr>
<td>29</td>
<td>9.20</td>
<td>9.78</td>
</tr>
<tr>
<td>30</td>
<td>9.99</td>
<td>10.05</td>
</tr>
<tr>
<td>31</td>
<td>10.16</td>
<td>9.68</td>
</tr>
<tr>
<td>32</td>
<td>10.16</td>
<td>9.97</td>
</tr>
<tr>
<td>33</td>
<td>10.31</td>
<td>10.48</td>
</tr>
<tr>
<td>35</td>
<td>10.67</td>
<td>10.69</td>
</tr>
<tr>
<td>36</td>
<td>10.29</td>
<td>10.15</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>10.11</td>
<td>10.11</td>
</tr>
</tbody>
</table>

Protein Residue, Main Lipid, and Aqueous Fractions

The data (Tables 2-4) on these fractions (2, 8, and 3, respectively) give further confidence in the method.

PLP

In the development of the procedure, much effort was devoted to the isolation of PLP. It was reasoned that if myelin is metabolically stable, as Davison believes (15), its proteins as well as lipids should have a low rate of turnover, and PLP, labeled during early development, should retain the label into adult life.* It was of primary importance, therefore, that the procedure should provide for the isolation of as much PLP as possible. In the first 20 experiments a modification of the original Folch purification procedure (29) was used and PLP was liberated by drying the lower phase under various conditions. The yields of PLP were small. Drying with acetone, as described at Step

*While this work was in progress, Davison (28) published data in accord with this prediction.
Table 4. PERCENTAGES OF WATER-SOLUBLE FRACTION (3) IN WET AND DRY TISSUE

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Percentage in wet tissue</th>
<th>Percentage in dry tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-1</td>
<td>H-2</td>
</tr>
<tr>
<td>29</td>
<td>1.52</td>
<td>1.57</td>
</tr>
<tr>
<td>30</td>
<td>1.47</td>
<td>1.50</td>
</tr>
<tr>
<td>31</td>
<td>1.50</td>
<td>1.50</td>
</tr>
<tr>
<td>32</td>
<td>1.49</td>
<td>1.46</td>
</tr>
<tr>
<td>33</td>
<td>1.43</td>
<td>1.42</td>
</tr>
<tr>
<td>35</td>
<td>1.44</td>
<td>1.42</td>
</tr>
<tr>
<td>36</td>
<td>1.48</td>
<td>1.47</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>1.48</td>
<td>1.48</td>
</tr>
</tbody>
</table>

F appeared to give the best results. In the remaining experiments, the technic of Webster and Folch (22) was applied to the extract from one of the half-brains (H-1); the lower phase from Step D (Fig. 5) was treated with a solution of citrate, or later, with a solution of NaCl as described at Steps E and F. With this procedure the total PLP is the sum of that released by the NaCl treatment (Fraction 4) and by drying (Fractions 6 and 7). For comparison, Steps E and F were not applied to the extract from the other half-brain (H-2); Step G was applied directly to the lower phase obtained from Step D, and the total PLP is that released by drying. The effect of NaCl treatment was thus tested in seven experiments. In three of these, carried out before the technic had been mastered, the data are suspect for various reasons. In the remaining four, the percentage of total PLP in dry tissue obtained with the NaCl treatment plus drying (H-1) exceeded that found with drying alone (H-2) by an average of 13.3% (range, 11.0-17.1). Of the total PLP obtained with procedure H-1, an average of 62.1% (range, 57.6-66.9) was released by the NaCl treatment.

Precision of Weighing

The procedure used at Step G affords an opportunity to evaluate the precision of weighing. The main lipid plus PLP fraction (Fraction 5 in the procedure as described, or the lower phase from Step D with procedure H-2) is divided into Fractions 6, 7, and 8, whose sum should equal the weight of the original fraction. In 14 such sets of weighings (two in each of the experiments listed in Tables 2–4), the average recovery was 100.06%, and the maximal deviation was 0.68%.

Completeness of Lipid Extraction

In many of the experiments, the protein residue (Fraction 2) was digested on a steam bath for several hours in a solution of approxi-
mately 3.5N KOH in 50% ethanol. After acidification and dilution with 50% ethanol, the solution was equilibrated with petroleum ether, the mixture was allowed to stand over night to clarify the phases, the upper phase was removed, and the lower phase was washed 3 times with petroleum ether. The combined petroleum ether solution was washed with 10% ethanol and transferred to a 50-ml Erlenmeyer flask. The solvent was removed with nitrogen, and the residue was dissolved in a little petroleum ether and transferred through a filter (Fig. 2D (19)) into a tared 5-ml volumetric flask, where it was dried and weighed. A residue equivalent to about 0.4% of the dry tissue was always found. It has not yet been investigated, but it behaves like fatty acid and certainly must represent lipid which is so firmly bound in the brain as to resist the exhaustive extraction procedures which were applied.

"Strandin" Fraction 9

In four of the experiments listed in Tables 2–4, the two Fractions 3 were pooled for Step H; in the others, each fraction was dialyzed separately. The average of the 10 measurements was 1.25% of Fraction 9 in dry tissue (range, 1.16–1.31). Analyses for sialic acid, hexosamine, hexose, and sphingosine indicated that the fraction is largely strandin, as suggested by its appearance (see above).

"Acid" Fraction 11

Much effort was devoted to this fraction with the primary objective of providing in the procedure for the isolation of the phosphatido-peptides shown by LeBaron and Folch (23) to be extracted by acidified C-M, or of the triphosphoinositides, found by Dittmer and Dawson (30) to be the major lipid component of these peptides. Many procedures based on the Folch purification technics were tried without success. A reason for this failure became evident with the publication by Dittmer and Dawson (31) of their detailed results. They isolated crude triphosphoinositide equivalent to 3.17–9 mg. of P from 200-gm. portions of ox brain. If rat brain contains corresponding concentrations, less than 0.5 mg. of triphosphoinositide would have been present in the amounts of tissue analyzed in this work, i.e., less than 3% of the weight of Fraction 11.

The early attempts to fractionate the acid extract failed also from another point of view; amorphous, intractable precipitates and emulsions were obtained. These difficulties were overcome in the procedure described in Steps I–M. It consistently yields the following four well-defined fractions.
Chloroform-soluble "Acid" Fraction 15

This fraction appears to contain most of the pigment of the tissue. In eleven determinations on half-brains its percentage in dry tissue averaged 0.77 ± 0.09 (S.D.). In five determinations* (four on pooled samples) its fatty acid content was 21.0 ± 2.4%, calculated as stearic acid.

Insoluble "Acid" Fractions 18, 19, and 20

These fractions almost certainly are composed mainly of protein. In two experiments the fractions were hydrolyzed in boiling 6N HCl, combined, dried in a rotary evaporator, and redried several times after addition of water to remove HCl. The residues, which were soluble in water, contained 10.4 and 11.2% nitrogen. In 9 half-brains the sums of the three fractions averaged 2.46 ± 0.33 (S.D.)% of dry tissue.

Nondialyzable Water-soluble "Acid" Fraction 21

In 9 half-brains, of which two were pooled for Step M, this fraction averaged 0.85 ± 0.15 (S.D.)% of dry tissue. (If one exceptionally low value—0.50%—is omitted, the average is 0.90 ± 0.06%.) This fraction dissolves instantly in water and is exceedingly surface active, so much so that it is difficult to handle in solution because of excessive foaming. Analyses of some of these fractions for a number of substances gave results so variable as to be meaningless. The reason is not known.

Dialyzable Water-soluble "Acid" Fraction 22

In 9 half-brains, of which two were pooled for Step M, this fraction averaged 3.40 ± 0.19 (S.D.)% of dry tissue. Analyses of some of these fractions gave the following average percentages (± S.D.): nitrogen, 5.0 ± 0.6; phosphorus, 4.9 ± 0.9; amino acids, expressed as serine, 13.2 ± 1.1; hexose, 2.0 ± 0.9; hexosamine, 0; fatty acids, 0; sialic acid, 0.5 ± 0.1.

Experiments with Acetate-C14

In accord with the purpose of this investigation (see above), at the start and a year later, rats within the first 16 days of postnatal life, were injected intraperitoneally with 31 or 33 μc of acetate-1-C14 or acetate-1,2-C14. Six of these rats were used in the experiments carried out in the development of the procedure; in these, the fractions iso-

*An unpublished method of Dr. Herbert L. Meltzer for determination of ester groups was used in these analyses.
lated from the brain were examined for radioactivity. The counting was done in an old, inefficient, end-window counter with the purpose of ascertaining qualitatively whether or not detectable C\textsuperscript{14} was present, but sufficient counts were recorded, usually for five 10-min. periods interspersed with an equal number of background counting periods, to permit statistical analysis of the results. The data will not be presented in detail here; only the principal findings will be summarized. The rats had received labeled acetate 5–15 months before they were used in these experiments. In all of them, most of the fractions examined contained significant radioactivity. The main lipid fraction, Fraction 8, was by far the most active, as expected, but the main protein fraction, Fraction 2, always showed a significant number of counts above background. It is not certain, however, that this radioactivity was in the protein and nucleic acids since the small fatty acid fraction isolated from Fraction 2 always was significantly radioactive.

The “strandin” fraction, Fraction 9, was consistently the most radioactive of all except the main lipid fraction, Fraction 8. This result was unexpected since the gangliosides are not components of myelin where, according to Davison’s hypothesis, the metabolically stable lipids of brain are. But the possibility has not been ruled out that the C\textsuperscript{14} of Fraction 9 was present in contaminating lipids from myelin.

Also surprising was the presence of little or no radioactivity in PLP, counted after hydrolysis of the combined fractions with 6N HCl and treatment of the hydrolysate as described for the insoluble “acid” fractions. The amount of material was small, but significant radioactivity was found consistently under the same conditions in considerably smaller fractions, particularly the chloroform-soluble “acid” fraction, Fraction 15, and the fatty acid fraction from the protein residue. The apparent discrepancy between this finding and that of Davison (28) may have resulted from a failure of PLP to be labeled by acetate-C\textsuperscript{14} (Davison used glycine-C\textsuperscript{14}), but this explanation is difficult to understand. The insoluble “acid” protein fractions, Fractions 18–20, consistently contained small but significant radioactivity. The only other fraction which was consistently free of significant radioactivity was the dialysate, Fraction 10.

The foregoing results are preliminary and subject to revision when a better counting procedure is applied, but they do substantiate the finding by Davison and his colleagues of a remarkable metabolic stability of some substances in the brain and they suggest that the num-
ber of such relatively inert compounds may be greater than has been thought. Perhaps such findings should have been anticipated from the work of Thompson and Ballou (32, 33), who found significant concentrations of tritium in all rat tissues studied, including brain, up to a year after exposure of the animals to $\text{H}_2\text{O}$.

References

33. Thompson, R. C., and Ballou, J. E., J. Biol. Chem. 223, 795 (1956).